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RESEARCH PAPER

The potential of secondary metabolites of *Trichoderma viride* T1sk extracted with organic solvents etil acetate and butanol in suppressing the growth of *Colletotrichum gloeosporioides* in vitro

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ABSTRACT

The control of *Colletotrichum gloeosporioides*, the causal agent of anthracnose in chili, is typically achieved through synthetic fungicides, which pose risks to both the environment and consumers. As a safer alternative, microbial-derived biopesticides offer an environmentally friendly solution. This study aimed to evaluate the potential of secondary metabolites produced by *Trichoderma viride* T1sk, extracted using organic solvents with different polarities (ethyl acetate and butanol), to inhibit the growth of *C. gloeosporioides* in vitro. A Completely Randomized Design (CRD) was used, comprising three treatments with six replications, each consisting of two petri dish units. The treatments included: (A) ethyl acetate extract, (B) butanol extract, and (C) a control. The media poisoning method was employed to assess the antifungal activity of the extracts. Key parameters observed were colony area, conidial count, conidial germination rate, and propagule density of *C. gloeosporioides*. The results demonstrated that both ethyl acetate and butanol effectively extracted antifungal secondary metabolites from *T. viride* T1sk. In general, these metabolites significantly suppressed the growth of *C. gloeosporioides*. Among the solvents tested, butanol exhibited the highest efficacy in extracting antifungal compounds and consistently achieving the strongest inhibition across all observed variables.

Key words: *Colletotrichum gloeosporioides*, organic solvent, secondary metabolites, *Trichoderma viride*

INTRODUCTION

Anthracnose disease, caused by *Colletotrichum gloeosporioides*, is a major threat to chili (*Capsicum annuum*) production, leading to significant yield losses ranging from 20% to 90% (Sutomo et al., 2022). This disease manifests as circular, sunken lesions with concentric rings, exhibiting a reddish-brown center and dark brown to blackish edges (Kim et al., 2004; Robert et al., 2008; Manda et al., 2020).

Synthetic fungicides are the primary means of controlling *C. gloeosporioides*, but their excessive use poses environmental hazards, leaves pesticide residues on crops, and restricts export opportunities. Additionally, prolonged exposure to these chemicals has been linked to health risks for farmers, particularly in developing countries (Voorrips et al., 2004; Tudi et al., 2021). Consequently, there is a growing need for eco-friendly alternatives, such as microbial-based biopesticides.

Trichoderma spp., well-known antagonistic fungi, offer a promising biocontrol strategy against plant pathogens, including foliar and fruit diseases. Although their use in controlling chili anthracnose has not been extensively documented, *Trichoderma* spp. are naturally occurring soil fungi that enhance plant growth and suppress pathogens. They improve crop yields, promote plant vigor, and produce a wide array of antimicrobial secondary metabolites (Iqbal & Mukhtar, 2020). Large-scale production of *Trichoderma* spp. is both cost-effective and environmentally sustainable.

As a saprophytic fungus, *Trichoderma* viride produces diverse secondary metabolites with antifungal properties, including peptaibols, polyketides, terpenes, and steroids (Cardoza et al., 2011; Khan et al., 2020). These metabolites can be classified as polar or nonpolar, influencing their solubility and bioactivity. Polar metabolites such as gliovirin and diketopiperazines generally have higher molecular weights, whereas nonpolar metabolites include polyketides (pyrones,

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isocyanates, butenolides) and volatile terpenes (Degenkolb et al., 2008; Reino et al., 2008; Mukherjee et al., 2012; Vinale & Sivasithamparam, 2020; Li et al., 2019; Lee et al., 2015). *T. viride* also produces viridine, a steroid known to inhibit fungal spore germination (Chet & Haran, 2005; Guzmán-Guzmán et al., 2023; Nurbailis et al., 2023). However, optimizing the growth conditions can maximize secondary metabolite production (Ming et al., 2012).

Microbial secondary metabolites are typically extracted from liquid cultures using organic solvents of varying polarities. The choice of solvent significantly influences the extraction efficiency, as different solvents selectively dissolve specific metabolites. Nonpolar solvents (e.g., n-hexane) extract lipophilic compounds, while polar solvents (e.g., ethanol, methanol) extract hydrophilic metabolites. Semipolar solvents, such as ethyl acetate and butanol, are particularly effective for isolating both polar and nonpolar bioactive compounds (Roy & Dehdulal, 2010; Conrado et al., 2022; Lefebvre et al., 2021; Yusnawan, 2013; Subandrate et al., 2024).

This study aimed to evaluate the potential of ethyl acetate and butanol as extraction solvents for *T. viride* T1sk secondary metabolites and their effectiveness in suppressing the growth of *C. gloeosporioides* in vitro.

MATERIALS AND METHODS

Research Site. The study was conducted from June to October 2021 at the Phytopathology Laboratory of the Department of Plant Protection, Faculty of Agriculture, and the Natural Materials Laboratory at Andalas University, Padang.

Experimental Design. The study used a Completely Randomized Design (CRD) consisting of three treatments and six replications, with each replication consisting of two petri dish units. The treatments included: Control = No secondary metabolite extract; Ethyl acetate extract = *T. viride* T1sk extracted with ethyl acetate; Butanol extract = *T. viride* T1sk extracted with butanol.

Fungal Isolates. The *T. viride* T1sk isolate was obtained from the rhizosphere of banana plants (Kepok cultivar) in Lima Kaum sub-district, Tanah Datar district, West Sumatra (-0.467967, 100.570104). *C. gloeosporioides* was isolated from infected chili (*Capsicum annuum*) fruits collected from a community plantation in Pauh sub-district, Padang City (-0.920354, 100.469943). The pathogen was identified based on morphological characteristics, and pure cultures were maintained on Potato Dextrose Agar (PDA) medium.

Fungal isolation was performed using the direct planting method. Infected chili fruits were sectioned into 1 cm × 1 cm pieces, including both symptomatic and asymptomatic tissues. Surface sterilization was conducted by sequentially immersing the tissue in sterile distilled water, 70% ethanol, and sterile distilled water for one minute each. The samples were then dried on tissue paper before being placed onto PDA in petri dishes using sterile tweezers, with each dish containing five tissue pieces. Fungal growth suspected to be *C. gloeosporioides* was transferred to fresh PDA medium and subcultured until a pure isolate was obtained. A single fungal colony was then incubated on PDA for 2 to 14 days (Harahap et al., 2013).

Preparation and Propagation of *T. viride* T1sk. To revive the isolate, a 0.5 cm diameter section of fungal culture was obtained using a cork borer and transferred to a 9 cm petri dish containing PDA medium (200 g potato, 20 g dextrose, 20 g agar, and 1000 mL distilled water). The petri dishes were incubated at room temperature for seven days.

For propagation, PDB medium was prepared using 200 g potato, 20 g dextrose, and 1000 mL distilled water. A 14 day-old pure culture of *T. viride* T1sk was suspended in 10 mL of sterile distilled water. Conidia were collected using a sterile brush, and the suspension was transferred to a test tube, then homogenized for one minute using a vortex mixer. The conidia concentration was determined using a hemocytometer and adjusted to 10^6 conidia/mL.

For cultivation, 250 mL of PDB medium was dispensed into a 500 mL Erlenmeyer flask. The medium was inoculated with *T. viride* T1sk at 1% its volume (10^6 conidia/mL). Cultures were incubated for two weeks at room temperature with continuous agitation at 100 rpm using an orbital shaker (DLAB SK-0330-Pro) (Vandermolen et al., 2013).

Extraction of Secondary Metabolites. A total of 1000 mL of *T. viride* T1sk culture in PDB medium was transferred to a 2.5-L opaque container and extracted with an equal volume of 100% ethyl acetate (Pro analysis grade) for 24 h. The mixture was then filtered using Whatman No. 1 filter paper to separate the organic phase. The residual *T. viride* T1sk biomass underwent a second maceration with another 1000 mL of ethyl acetate for 24 h. The combined ethyl acetate extracts were concentrated using a Rotary Evaporator RE-1000 VN B-ONE.

Next, the remaining ethyl acetate fraction and

filtered *T. viride* T1sk biomass were macerated with 1 L of butanol for 24 hours. The butanol extract was then concentrated via rotary evaporation. The final ethyl acetate and butanol extracts were collected for further analysis and experimentation.

Rejuvenation of *C. gloeosporioides*. The *C. gloeosporioides* isolate was subcultured on PDA medium and incubated for 14 days at room temperature before being used in the experiment.

Antifungal Assay Using the Poisoned Food Technique. The antifungal activity of *T. viride* secondary metabolite extracts was evaluated using the poisoned food technique. A 10% concentration of each extract was prepared by dissolving 1 g of extract in 9 mL of sterile distilled water. The extract was sterilized using a 0.2 µm membrane filter. Then, 1 mL of each extract was mixed with 9 mL of molten PDA medium (45°C) before being poured into petri dishes. Once solidified, a 5 mm fungal disc of *C. gloeosporioides* was placed at the center of each plate. The plates were incubated at room temperature, and colony growth was measured daily until the control plates were fully covered.

Observation and Data Collection

Colony Area Measurement. The area of *C. gloeosporioides* colonies was measured from the third to the twelfth day after inoculation using transparent plastic sheets and graph paper.

***Conidial Production*.** A 10 mL suspension of *C. gloeosporioides* conidia was collected and adjusted to a known dilution. The number of conidia per mL was determined using a hemocytometer under a light microscope. The conidial density was calculated following Lacey's (1997) formula:

$$C = R \times 2.5 \times 10^5 \times P$$

C = Spore density (conidia/mL);
R = Average conidia;
P = Dilution factor;
0.25×10^6 = Correction factor.

Conidial Germination Rate. Conidial germination was determined according to the method proposed by Junianto & Sukamto (1995). Germination assays were performed on water agar (WA) medium. A 10 µL conidial suspension (10^3 conidia/mL) was placed on a WA-coated microscope slide, incubated for 24 hours, and examined under a light microscope at 400× magnification. The germination percentage was calculated. The germination rate was calculated using the following formula (Ramadani et al., 2023):

$$V = \frac{g}{(g + u)} \times 100\%$$

V = Conidial germination rate;
g = Number of germinating conidia;
u = Number of non-germinating conidia.

Data Analysis. Data were analyzed using analysis of variance (ANOVA) in the Statistix 9.0 program, followed by a Least Significant Difference (LSD) test at a 5% significance level to determine differences between treatments.

RESULTS AND DISCUSSION

Colony Growth of *C. gloeosporioides.* The results demonstrated that the secondary metabolites extracted from *Trichoderma viride* T1sk significantly inhibited the colony growth of *C. gloeosporioides*. Colonies treated with ethyl acetate and butanol extracts showed significantly smaller than the control (Table 1). Between the two solvents, butanol extract exhibited the highest antifungal activity, effectively suppressing colony expansion more than ethyl acetate (Figure 1).

In the control treatment, colony growth increased rapidly from day 3 to day 12 without inhibition. Meanwhile, colonies terated with ethyl acetate solvent expanded until day 9, after which growth remained constant. This may be due to the semi-polar solvent of ethyl acetate, which extract both polar and non-polar secondary metabolites from *T. viride.* Non-polar compounds are more volatile and likely evaporated by day 9, becoming trapped in the petri dish and restricting further fungal growth (Rao & Sunkada, 2007).

In the butanol solvent, colony expansion was initially slow, increasing after day 4 and continuing until the end of the observation period. Butanol, a polar solvent, extracts polar secondary metabolites from *T. viride*, which effectively inhibited stunted growth compared to the control (Figure 2), confirming the antifungal properties of *T. viride* secondary metabolites (Figure 2).

Trichoderma spp. produce various non-polar secondary metabolites, such as pyrones, steroids, terpenoids, and polyketides, which dissolve in ethyl acetate and exhibit antifungal properties (Zeilinger et al., 2016). Additionally, *Trichoderma* spp. synthesize polar compounds, including glyceryl 1-oleate diacetate,

Table 1. Colony growth of *C. gloeosporioides* as affected by secondary metabolite extracts from *T. viride*

Treatments	Colony area (mm^2)	Effectiveness (%)
Control	34.30 a ± 0.72	00.00
Ethyl acetate	20.60 b ± 0.15	39.94
Butanol	17.87 b ± 0.40	47.90

The number followed by the same letter on the same column are not significantly different at the 5% level by LSD.

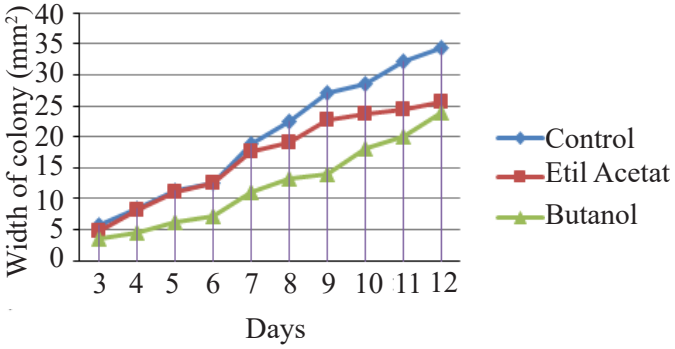

Figure 1. Growth inhibition of *C. gloeosporioides* fungal colonies in response to secondary metabolite extracts from *T. viride* T1sk, measured from the third day to twelfth day after inoculation.

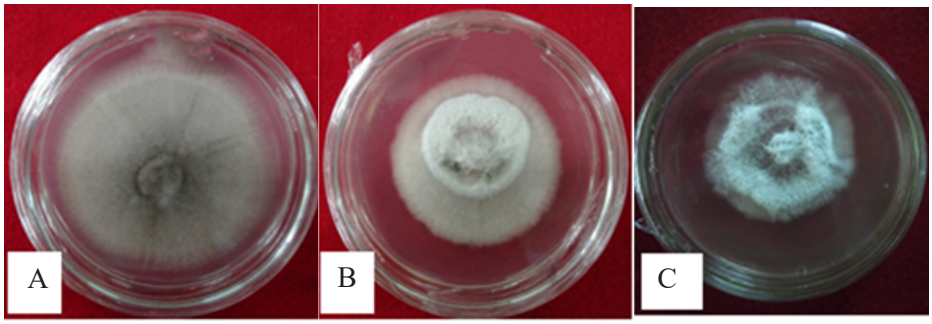

Figure 2. The growth of *C. gloeosporioides* 12 days after inoculation as affected by secondary metabolite extracts from *T. viride*. A. Control; B. Ethyl acetate solvent; C. Butanol solvent.

monolinolein, TMS, and erucylamide, which also contribute to antifungal activity (Lakhdari et al., 2023). Thus, the combined action of polar and non-polar metabolites plays a crucial role in fungal suppression.

Fungal inhibition in butanol and ethyl acetate treatments resulted in hyphal clumping (Figure 2). This occurs because secondary metabolites disrupt disulfide bonds in fungal cell walls and membrane proteins, leading to structural instability. The breakdown of these bonds weakens the cell wall, making fungal cells more susceptible to external stress. Additionally, membrane protein denaturation compromises cell permeability, inhibiting the selective transport of substances. This ultimately allows secondary metabolites to penetrate the cells, causing lysis and death (Harni et al., 2017). Phenolic and flavonoid compounds, which are commonly found in secondary metabolites, further contribute to antifungal activity by altering microbial cell permeability and disrupting membrane protein functions (Zaynab et al., 2018).

Conidial Production and Germination of *C. gloeosporioides*. Conidial production and germination rates were significantly affected by *T. viride* extracts (Table 2). The number of conidia in both butanol and ethyl acetate treatments was lower than in the control, with butanol exhibiting the strongest inhibitory effect. This suggests that butanol-soluble secondary metabolites interfere with fungal sporulation, preventing viable conidia formation.

Conidial germination was also significantly reduced, with butanol extract demonstrating stronger suppression than ethyl acetate. This aligns with previous findings that secondary metabolites such as gliotoxin and peptaibols disrupt fungal spore germination by targeting cellular respiration and

Table 2. Conidial production and germination of *C. gloeosporioides* as affected by various secondary metabolite extracts from *T. viride*

Treatments	Conidial production (conidia/mL)	Effectiveness (%)	Conidia germination (%)	Effectiveness (%)
Control	3.75×10^8 a	0.00	60.00	0.00
Ethyl acetate	1.25×10^8 b	66.67	45.00	25.00
Butanol	0.25×10^8 b	93.33	37.00	38.33

The number followed by the same letter on the same column are not significantly different at the 5% level by LSD.

oxidative stress pathways (Howell, 1999; Vinale et al., 2014). Additionally, tannins in *Trichoderma* extracts enhance antifungal activity by inhibiting enzymatic processes essential for germination (Lakhdari et al., 2023).

Viridin, an antifungal compound isolated from *T. koningii*, *T. viride*, and *T. virens,* has been reported to inhibit spore germination in *Botrytis allii*, *Colletotrichum lini*, *Fusarium caeruleum*, and *Aspergillus niger* (Vinale et al., 2014). Similarly, tannins disrupt fungal cell wall permeability, impairing metabolic processes (Hersila et al., 2023). The efficacy of secondary metabolites depends on extraction methods, as different solvents influence the total yield and composition of bioactive compounds due to their varying polarities (Dirar et al., 2019).

Mechanism of Growth Inhibition. The inhibition of *C. gloeosporioides* by *T. viride* extracts is attributed to the presence of multiple secondary metabolites with antifungal properties. Ethyl acetate, a semi-polar solvent, extracts both polar and non-polar compounds, whereas butanol primarily extracts polar bioactive compounds. The stronger inhibition observed with butanol extract suggests that polar metabolites, such as diketopiperazines and certain alkaloids, play a crucial role in fungal suppression (Mukherjee et al., 2012; Simorangkir et al., 2019).

Implications for Biocontrol Applications. This study demonstrate the potential of *T. viride* secondary metabolites as effective biocontrol agents against *C. gloeosporioides*. The use of butanol as an extraction solvent yielded the most potent antifungal effects, suggesting that further research should focus on identifying and purifying the specific active compounds responsible for this activity.

Future studies should investigate the field application of *T. viride* extracts in managing anthracnose disease in chili plants. Additionally, the formulation and stability of these extracts need to be assessed to enhance their practical use in agricultural settings. The findings support the broader implementation of *T. viride*-based biopesticides as an eco-friendly alternative to synthetic fungicides.

CONCLUSION

The results of this study showed that, in general, secondary metabolites dissolved in ethyl acetate and butanol could suppress the growth of *C. gloeosporioides*. Butanol was the most effective solvent for extracting the antifungal compounds of *T. viride,* and it successfully inhibited *C. gloeosporioides* growth across all observed variables.

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AUTHORS' CONTRIBUTIONS

N coordinated and conducted the research, including observations, data collection, analysis, and manuscript writing. YY contributed to data collection, analysis, and manuscript editing. AJ supervised the extraction of secondary metabolites using organic solvents and assisted with data analysis. SDR provided laboratory support, including testing pathogen

inhibition by secondary metabolite extracts of *T. viride*

COMPETING INTEREST

The authors declare no competing interests in this publication.

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